of glacial acetic acid and 10 g. of zinc dust. The mixture was refluxed under nitrogen for 3 hr. After the usual work-up, bases were extracted with ether. The resulting mixture was crystallized from methanol to give 510 mg. of carbomethoxydihydrocleavamine.[4,26] The hydrochloride was recrystallized from acetone, m.p. 220–224° dec.

Anal. Calcd. for $C_{21}H_{28}N_2O_2 \cdot HCl$; C, 66.91; H, 7.78; N, 7.43. Found: C, 66.97; H, 7.84; N, 7.97.

Dihydrocleavamine (XII). Cleavamine base was hydrogenated using platinum oxide in ethanol at atmospheric pressure for 2 days. After the usual work-up, the base was recrystallized from aqueous methanol, m.p. 136–138°, $pK_a' = 8.8$, $[\alpha]^{26}D$ −67.6°.

Anal. Calcd. for $C_{19}H_{26}N_2$: C, 80.80; H, 9.28; N, 9.92. Found: C, 80.56; H, 9.27; N, 9.87.

Epimerization of Dihydrocatharanthine. A. Hydrochloric Acid. A mixture of 100 mg. of dihydrocatharanthine hydrochloride in 10 ml. of concentrated hydrochloric acid was refluxed under nitrogen for 16 hr. after which time the solution was homogeneous. The reaction mixture was evaporated *in vacuo* to dryness and worked up as usual for the free base. Chromatography on alumina using 1:1 petroleum ether–benzene as eluent afforded 37 mg. of ibogamine and 32 mg. of epiibogamine (infrared and X-ray diffraction patterns).

B. Acetic Acid. Dihydrocatharanthine (180 g.) was refluxed with 3240 ml. of glacial acetic acid for 42 hr., evaporated *in vacuo* to a sirup, taken up in 2 l. of water, filtered, made alkaline, and extracted with ether. After evaporation, the residue (90 g.) contained about equal amounts of the starting material and coronaridine (t.l.c.). Chromatography on Mallinckrodt silicic acid (ratio 10:1) in benzene afforded, after elution with 40 l. of benzene, 34 g. of coronaridine. Continued elution with chloroform–benzene (25:75) gave 25 g. of dihydrocatharanthine.

C. Zinc and Acetic Acid.[26] Zinc dust (15 g.) was added to a solution of amorphous dihydrocatharanthine (0.45 g.) in glacial acetic acid (30 ml.). The solution was refluxed 3 days, removing aliquots after 4 hr. and after 1 day; t.l.c. of the aliquots and of the product indicated the presence of a trace of coronaridine and a slower moving material which increased with time. After 3 days, two other products, moving in the same area as dihydrocatharanthine, were evident with less than 20% dihydrocatharanthine remaining. The residue was chromatographed on alumina (10 g.), using benzene as an eluent. The second fraction (175 mg.) contained several components on t.l.c. and was rechromatographed on silicic acid (4 g.) in chloroform. The first two fractions afforded 53 mg. of material which crystallized from methanol and was shown to be carbomethoxydihydrocleavamine.[4]

Decarboxylation of Carbomethoxydihydrocleavamine. Preparation of Epidihydrocleavamine. A solution of 3.2 g. of carbomethoxydihydrocleavamine in 100 ml. of ethanol containing 15 g. of potassium hydroxide in 20 ml. of water was boiled under reflux for 4 hr. After dilution with water, concentrated hydrochloric acid was added to pH 2.2, and the solution was boiled under reflux for 50 min. After cooling and addition of ammonium hydroxide followed by extraction with methylene dichloride, 2.8 g. of base was obtained. Chromatography on 90 g. of alumina (activity III) using benzene as eluent afforded 1.94 g. of material in the first fraction. It crystallized from methanol and afforded 1.2 g. of epidihydrocleavamine, m.p. 109–111°, [M] = 282, $[\alpha]^{26}D$ +94.3, $pK_a' = 9.0$.

Anal. Calcd. for $C_{19}H_{26}N_2$: C, 80.80; H, 9.28; N, 9.92. Found: C, 80.75; H, 9.61; N, 10.05.

Acknowledgment. We gratefully acknowledge the assistance of the following individuals in the course of this work: physical data, Dr. Harold Boaz, Messrs. Paul Landis, Lee G. Howard, Donald O. Woolf, and their associates; microanalyses, Mr. George M. Maciak and his associates; catharanthine isolation: Mr. Al Barnes and his associates; mass spectra, Professor Klaus Biemann, Department of Chemistry, Massachusetts Institute of Technology.

New Methods in Peptide Synthesis. II. Further Examples of the Use of the *o*-Nitrophenylsulfenyl Group for the Protection of Amino Groups[1,2]

Leonidas Zervas and Charalambos Hamalidis

Contribution from the Laboratory of Organic Chemistry, University of Athens, Athens, Greece. *Received August 31, 1964*

Further examples are presented of the usefulness of the N-o-nitrophenylsulfenyl (NPS) group as an N-protecting group for α-amino acids or α-amino acids bearing a protected side-chain functional group. The wide applicability of the new method is demonstrated by the synthesis of various peptides, e.g., L-*phenylalanyl*-L-*glutaminyl*-L-*glutamyl*-L-*glutamine, which normally present great difficulties in their synthesis by conventional methods.*

(1) A summary of this paper was presented at the 6th European Peptide Symposium, Athens, Greece, Sept. 1963; E. Gazis, D. Borovas, C. Hamalidis, G. C. Stelakatos, and L. Zervas in "Peptides: Proceedings of the Sixth European Peptide Symposium, Athens, 1963," Pergamon Press, Oxford, 1964, in press.

(2) This investigation was supported by the Royal Hellenic Research Foundation, to which we are greatly indebted.

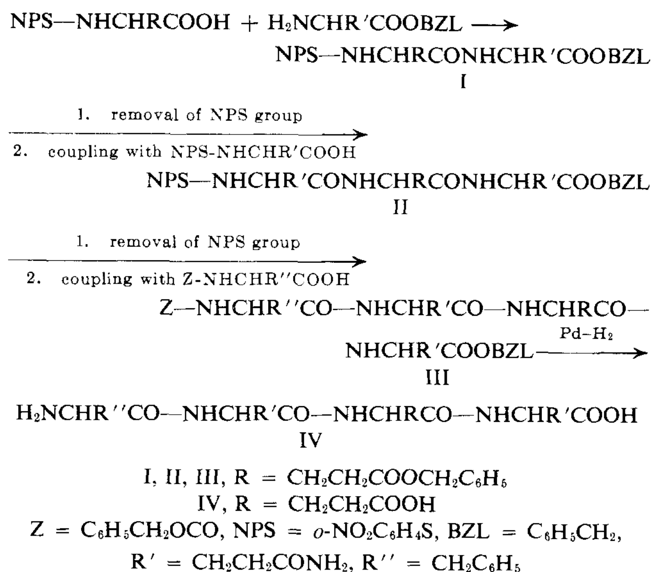
Introduction

In the first publication of this series,[3] the use of the *o*-nitrophenylsulfenyl (NPS) group for the N-protection of amino acids during peptide synthesis was reported. In this paper, new examples of such protection are presented which demonstrate the wide applicability of this method (sulfenyl method).

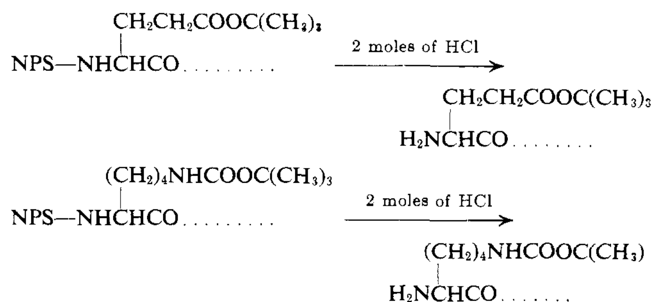
Treatment of L-cystine and L-lysine with *o*-nitrophenylsulfenyl chloride in aqueous alkaline solution in the manner already described[3] afforded the corresponding N,N′-di-NPS derivatives. By the same procedure, L-serine and its methyl ester were transformed into their corresponding N-NPS derivatives, whereas L-cysteine methyl ester formed the S,N-di-NPS derivative. The preparation of cystine peptides *via* the sulfenyl method is described in the Experimental part.

The usefulness of the NPS-protecting group becomes more apparent in the case of the incorporation into a peptide chain of amino acids bearing functional groups in their side chains. These functional groups can be protected, for instance, with benzyl or *t*-butyl groups,[4] as in the case of glutamic acid, aspartic acid, serine, cysteine, histidine, etc., or with carbobenzoxy, *t*-butyloxycarbonyl, and trityl groups[4,5] (as in the case of lysine, arginine, and histidine), or with acyl groups,[6] as in the case of cysteine. It has been found that side-chain-protecting groups, such as N- and S-trityl[7] and N-carbobenzoxy groups,[3] are unaffected by the mode of removal of the N-NPS group, thus allowing the side-chain-substituted amino acids to be incorporated into a peptide chain by means of the sulfenyl method. An example of this type of synthesis is the preparation of L-prolyl-N$^{\epsilon}$-carbobenzoxy-L-lysyl-L-alanine methyl ester, which has been included in the first publication of this series.[3] In order to broaden our experience in this field, we have prepared the N-NPS derivatives of α- and γ-benzyl L-glutamate, β-benzyl L-aspartate, N$^{\omega}$-carbobenzoxy-L-arginine, and S-benzoyl- and S-carbobenzoxy-L-cysteine. Many of these derivatives, in turn, have been used for the synthesis of peptides such as S-benzoyl- (and S-carbobenzoxy-) L-cysteinylglycine methyl ester (*cf.* Experimental part) and of the protected tetrapeptide benzyl carbobenzoxy-L-phenylalanyl-L-glutaminyl-γ-benzyl-L-glutamyl-L-glutaminate (III). After the formation of the peptide chain, the removal of the side-chain-protecting groups can be easily accomplished using well-established methods. In the case of the protected peptide III, the free tetrapeptide IV can be obtained by splitting off all the protecting groups by hydrogenolysis in the usual way.

Of special importance, in our opinion, is the use of amino acids bearing the *t*-butyl group in the side chain; for example, γ-*t*-butyl L-glutamate and N$^{\epsilon}$-*t*-butyloxycarbonyl-L-lysine. The O-*t*-butyl groups not only



survive the conditions for the removal of the NPS group, but also can be subsequently split off under relatively mild conditions, *i.e.*, by shaking for a few minutes with trifluoroacetic acid.[8] Since almost all the above N-, O-, and S-protecting groups (S- or N-



carbobenzoxy, S- or O-benzyl, S-acyl, S- or N-trityl, O-*t*-butyl, or N-*t*-butyloxycarbonyl groups) are not sensitive toward low concentrations of hydrogen chloride in nonpolar solvents, they do not, consequently, interfere in the lengthening of a peptide chain by the sulfenyl method. Therefore, they can be used without any hesitation for the protection of the side chain.

Experimental

For the coupling reactions, anhydrous reactants and dry solvents were used; the ether was free of peroxides. Evaporations were carried out *in vacuo* at 35–40° The melting points are not corrected.

Prior to analysis[9] the compounds were dried at room temperature under high vacuum over phosphorus pentoxide.

β-Benzyl L-*Aspartate.* A solution of 7.15 g. (0.02 mole) of β-benzyl N-carbobenzoxy-L-aspartate[10] in 15 ml. of 4 *N* HBr in acetic acid was allowed to stand at room temperature for 15 min. The hydrobromide of the above substance precipitated out upon addition of dry ether. From a solution of this salt in 40 ml. of water, the above free ester separated after the addition

(3) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).
(4) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961.
(5) *Cf.* ref. 9 and 10 in L; Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962), as well as ref. 17–19 in L. Zervas, D. Borovas, and E. Gazis, *ibid.*, **85**, 3660 (1963).
(6) L. Zervas, I. Photaki, and N. Ghelis, *ibid.*, **85**, 1337 (1963).
(7) Both N$^{\epsilon}$-trityl-N$^{\alpha}$-NPS-L-lysine and S-trityl-N-NPS-L-cysteine, on treatment with 2 equiv. of HCl in ethyl acetate solution, give almost quantitatively the corresponding NPS-free derivatives.
(8) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **44**, 1136 (1961).
(9) Microanalyses were carried by Dr. H. Mantzos in the Analytical Laboratory of the Royal Hellenic Research Foundation.
(10) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **73**, 4084 (1951).

of 3.6 g. of the trihydrate of sodium acetate. After recrystallization of the product from water, the yield was 2 g. (45%), m.p. 212°, $[\alpha]^{15}D$ +28.6° (*c* 1, 1 *N* HCl); lit.[11] m.p. 218–220°, $[\alpha]D$ +29.1° (*c* 1, 1 *N* HCl).

*β-Benzyl α-p-Nitrophenyl N-Carbobenzoxy-*L*-Aspartate.* To a solution of 7.2 g. of β-benzyl N-carbobenzoxy-L-aspartate[10] and 3.1 g. of *p*-nitrophenol in 100 ml. of ethyl acetate, 4.4 g. (10% excess) of N,N′-dicyclohexylcarbodiimide was added. After standing at room temperature overnight, the insoluble precipitate of dicyclohexylurea was filtered, and the filtrate was evaporated to dryness. Upon recrystallization of the crystalline residue from ethanol, 8.2 g. (85%) of the product was obtained, m.p. 75–76°, $[\alpha]^{30}D$ +12.5° (*c* 4, chloroform).

Anal. Calcd. for $C_{25}H_{22}O_8N_2$: C, 62.75; H, 4.64; N, 5.85. Found: C, 62.55; H, 4.75; N, 5.76.

β-Benzyl α-p-Nitrophenyl L*-Aspartate Hydrobromide.* A solution of 2.4 g. (0.005 mole) of β-benzyl α-*p*-nitrophenyl N-carbobenzoxy-L-aspartate in 4.5 ml. of warm acetic acid was allowed to cool to room temperature; 2.5 ml. of 6.5 *N* HBr in acetic acid was added, and the solution was kept for 20 min. at room temperature. Addition of ether and cooling caused the hydrobromide to separate. The yield was 1 g. (47%), m.p. 135–137° after recrystallization from acetone–ether, $[\alpha]^{25}D$ +38.9° (*c* 4, ethanol).

Anal. Calcd. for $C_{17}H_{17}O_6N_2Br$: C, 48.02; H, 4.03; N, 6.58; Br, 18.79. Found: C, 47.89; H, 4.16; N, 6.67; Br, 19.09.

γ-t-Butyl L*-Glutamate.* α-Benzyl γ-*t*-butyl N-carbobenzoxy-L-glutamate[12] (4.3 g., 0.01 mole) was catalytically (Pd) hydrogenated in methanol–water (2:1). The filtrate was evaporated to dryness; the residue was triturated with acetone and left in the refrigerator; 1.8 g. (90%) of the above crystalline substance was obtained, m.p. 182°, $[\alpha]^{27}D$ +9.82° (*c* 2, water); lit.[13] m.p. 168–169°, $[\alpha]D$ +8.15° (water).

Benzyl L*-Glutaminate Hydrochloride.* To a solution of 4.7 g. (0.01 mole) of benzyl N-trityl-L-glutaminate[14] in 20 ml. of ethyl acetate, 10 ml. of 1 *N* HCl in ether and, thereafter, 60 ml. of ether were added. The benzyl N-trityl-L-glutaminate hydrochloride thus formed was filtered and was dissolved in 10 ml. of absolute methanol. The solution was boiled for 2 min. on the steam bath. Upon addition of ether, crystalline benzyl L-glutaminate hydrochloride separated. The yield, after recrystallization from methanol–ethyl acetate, was 2 g. (82%), m.p. 120–122°, $[\alpha]^{20}D$ +13.0° (*c* 4, methanol).

Anal. Calcd. for $C_{12}H_{17}O_3N_2Cl$: C, 52.85; H, 6.28; N, 10.27; Cl, 13.00. Found: C, 52.87; H, 6.50; N, 10.12; Cl, 13.13.

*N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyl-*L*-lysine.* To a suspension of 5.6 g. (0.02 mole) of N^{α}-carbobenzoxy-L-lysine[15] in 24 ml. of water and 30 ml. of dioxane, 1.04 g. of magnesium oxide and 4.3 ml. of *t*-butyloxycarbonyl azide were added with stirring. The mixture was stirred for 12 hr. while the temperature was kept at 45–50°. The clear solution was allowed to stand at room temperature for an additional period of 12 hr. and then concentrated *in vacuo* until most of the dioxane was removed. The solution was acidified with dilute sulfuric acid and extracted twice with ethyl acetate. The ethyl acetate layer was extracted twice with dilute potassium hydrogen carbonate. Upon acidifying the combined aqueous extracts with sulfuric acid, extracting with ethyl acetate, washing the ethyl acetate extract repeatedly with water until the aqueous layer became neutral to congo red paper, drying over sodium sulfate, and evaporating to dryness, 6.2 g. (81%) of sirupy[16] product was obtained.

Upon the addition of dicyclohexylamine to an ethereal solution of the above sirup, the corresponding crystalline salt, m.p. 153–154°, separated in almost quantitative yield.

Anal. Calcd. for $C_{31}H_{51}O_6N_3$: C, 66.28; H, 9.15; N, 7.48. Found: C, 66.18; H, 9.15; N, 7.70.

*N^{ϵ}-t-Butyloxycarbonyl-*L*-lysine.* The sirupy N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyl-L-lysine (6.2 g., 0.016 mole), obtained by the method described above, was catalytically (Pd) hydrogenated in methanol containing 2 ml. of acetic acid, as usual. The product which separated during the hydrogenolysis was redissolved by adding 16 ml. of 1*N* HCl. The catalyst was filtered, and 2.2 g. of the trihydrate of sodium acetate was added to the filtrate. After concentrating *in vacuo* to a small volume, 2.8 g. (69%) of crystalline product was obtained, m.p. 238°, $[\alpha]^{24}D$ +14.4° (*c* 2.5, 0.1 *N* HCl); lit.[16] m.p. 237–255°, $[\alpha]^{26}D$ +4.7 ± 1° (*c* 0.882, 2 *N* NH_3).

*N-o-Nitrophenylsulfenyl-*L*-serine Methyl Ester.* To a solution of 1.55 g. (0.01 mole) of serine methyl ester hydrochloride in 10 ml. of chloroform and 2.8 ml. of triethylamine, 1.9 g. (0.01 mole) of *o*-nitrophenylsulfenyl chloride was added. After standing at room temperature for 2 hr., the solution was washed with water, dried over sodium sulfate, and evaporated to dryness. The crude product was recrystallized from methanol to yield 1.2 g. (43%), m.p. 136–138°, $[\alpha]^{25}D$ +45.3° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{10}H_{12}O_5N_2S$: N, 10.28; S, 11.74. Found: N, 10.12; S, 11.89.

*N,S-Di-o-nitrophenylsulfenyl-*L*-cysteine methyl ester* was prepared from 0.01 mole of cysteine methyl ester hydrochloride and 0.02 mole of *o*-nitrophenylsulfenyl chloride in the manner described above. The crude product was recrystallized from ethyl acetate; the yield was 20%, m.p. 198°.

Anal. Calcd. for $C_{16}H_{15}O_6N_3S_3$: N, 9.51; S, 21.78. Found: N, 9.79; S, 21.79.

N-o-Nitrophenylsulfenylamino acids were prepared by the interaction of *o*-nitrophenylsulfenyl chloride and α-amino acids, or α-amino acids bearing a protected side-chain functional group (for instance, α-benzyl L-glutamate,[14] γ-*t*-butyl L-glutamate, β-benzyl L-aspartate, N^{ϵ}-*t*-butyloxycarbonyl-L-lysine, N^{ω}-carbobenzoxy-L-arginine,[17] S-benzoyl-L-cysteine,[6] and S-carbobenzoxy-L-cysteine[18]), in aqueous alkaline solution by pro-

(11) L. Benoiton, *Can. J. Chem.*, **40**, 570 (1962).
(12) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).
(13) E. Taschner, A. Chimiak, J. F. Biernat, C. Wasielewski, and T. Sokolowska, *Ann.*, **663**, 188 (1963).
(14) C. Coutsogeorgopoulos and L. Zervas, *J. Am. Chem. Soc.*, **83**, 1885 (1961).
(15) B. Bezas and L. Zervas, *J. Am. Chem. Soc.*, **83**, 719 (1961).
(16) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).
(17) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
(18) A. Berger, J. Niguchi, and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).

Table I. N-*o*-Nitrophenylsulfenyl (NPS) Derivatives of Amino Acids

N^{α}-*o*-Nitrophenylsulfenyl derivatives of	Yield, %	M.p., °C.	$[\alpha]D$, deg.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found
L-Serine dicyclohexylammonium (DCHA) salt	61[a]	171–173[h]	−89[b]	$C_{21}H_{33}O_5N_3S$	9.56	9.46	7.26	7.06
L-Cystine DCHA salt[c]	66[d]	150[i]		$C_{42}H_{64}O_8N_6S_4$	9.24	9.22	14.20	13.92
L-Lysine DCHA salt[c]	43[a]	158–160[h]	−26.2[e]	$C_{30}H_{43}O_6N_5S_2$	11.05	11.00	10.12	10.39
β-Benzyl L-aspartate DCHA salt	70[a]	165–166[h]	−38.0[e]	$C_{29}H_{39}O_6N_3S$	7.53	7.61	5.75	5.79
α-Benzyl L-glutamate DCHA salt	70[a]	154[j]	−25.9[e]	$C_{30}H_{41}O_6N_3S$	7.36	7.36	5.60	5.25
γ-*t*-Butyl L-glutamate DCHA salt	88[d]	179–180[j]	−25.0[f]	$C_{27}H_{43}O_6N_3S$	7.81	7.83	5.96	5.81
N^{ϵ}-*t*-Butyloxycarbonyl-L-lysine DCHA salt	74[d]	194–195[h]	−43.4[g]	$C_{29}H_{48}O_6N_4S$	9.64	9.55	5.52	5.52
S-Benzoyl-L-cysteine DCHA salt	36[a]	168–169[h]	+30.2[e]	$C_{28}H_{37}O_5N_3S_2$	7.50	7.55	11.44	11.57
S-Carbobenzoxy-L-cysteine DCHA salt	50[a]	171[h]	−21.7[e]	$C_{29}H_{39}O_6N_3S_2$	7.12	7.13	10.87	10.93
N^{ω}-Carbobenzoxy-L-arginine[k]	90[l]	105		$C_{20}H_{23}O_6N_5S$	15.18	15.05	6.94	6.76

[a] Yield by method C. [b] *c* 1, in dimethylformamide. [c] N,N′-Di-NPS derivative. [d] Yield by method B. [e] *c* 4, in chloroform. [f] *c* 4, in methanol. [g] *c* 1.5, in chloroform. [h] After recrystallization from ethanol. [i] After trituration with hot dimethylformamide. [j] After recrystallization from ethyl acetate. [k] Prepared in this laboratory by D. Borovas. The N^{ω}-carbobenzoxy-L-arginine was prepared (84% yield) by S. G. Cottis upon treating N^{α},N^{ω}-dicarbobenzoxy-L-arginine for 30 min. with 2 *N* HBr in acetic acid at room temperature. The melting point, 186–187°, was not depressed when mixed with authentic N^{ω}-carbobenzoxy-L-arginine, prepared by the method described previously.[17] [l] Yield by method A.

cedure A, B, or C as described earlier.[3] The *o*-nitrophenylsulfenylamino acids thus prepared were isolated in the form of their dicyclohexylammonium (DCHA) salts, and are listed in Table I.

γ-t-Butyl L-*Glutamate Hydrochloride.* A suspension of 1.07 g. (0.002 mole) of γ-*t*-butyl N-*o*-nitrophenylsulfenyl-L-glutamate DCHA salt in ether was shaken in a separatory funnel with 15 ml. of 0.2 *N* sulfuric acid until the solid dissolved. The ether layer was separated and washed repeatedly with water until the washings were neutral to congo red paper. It was then dried over sodium sulfate and evaporated to dryness. The residue, consisting of crystalline γ-*t*-butyl N-*o*-nitrophenylsulfenyl-L-glutamate, was dissolved in 5 ml. of 1.5 *N* HCl in ether. Upon cooling and scratching, crystalline γ-*t*-butyl L-glutamate hydrochloride separated in almost quantitative yield; m.p. 180° dec., $[\alpha]^{25}D$ +22.6° (*c* 5, water).

Anal. Calcd. for $C_9H_{10}O_4NCl$: N, 5.84; Cl, 14.79. Found: N, 5.70; Cl, 14.88.

Upon removal of the HCl with silver oxide in the usual manner, free γ-*t*-butyl L-glutamate was obtained, m.p. 182°, $[\alpha]D$ +9.8° (*c* 2, water).

*N^{ϵ}-t-Butyloxycarbonyl-*L-*lysine Hydrochloride.* N^{ϵ}-*t*-Butyloxycarbonyl-N^{α}-*o*-nitrophenylsulfenyl-L-lysine DCHA salt (0.58 g., 0.001 mole) was transformed to the corresponding free acid in the manner described above. The sirupy N^{ϵ}-*t*-butyloxycarbonyl-N^{α}-NPS-L-lysine thus obtained was dissolved in a mixture of 3 ml. of acetone and 0.22 ml. of 5 *N* HCl. After allowing the solution to stand at room temperature for 20 min., it was evaporated to dryness and the residue was triturated with ether. The crystalline hydrochloride thus formed was filtered and was washed with ether. The yield was 0.23 g. (82%), m.p. 230–232°, $[\alpha]^{20}D$ +11.7° (*c* 2, water), R_f 0.54 (thin layer chromatography[19] in *t*-butyl alcohol–*n*-butyl alcohol–water[12]).

Anal. Calcd. for $C_{11}H_{23}O_4N_2Cl$: C, 46.72; H, 8.20; N, 9.91; Cl, 12.54. Found: C, 46.96; H, 7.92; N, 9.64; Cl, 12.25.

For comparison purposes, the above hydrochloride was also prepared by dissolving N^{ϵ}-*t*-butyloxycarbonyl-L-lysine in a small volume of methanol containing 1 equiv. of HCl. Upon the addition of ethyl acetate, the corresponding crystalline hydrochloride separated in almost quantitative yield; m.p. and m.m.p. 230–232°, $[\alpha]D$ +11.9° (water), R_f 0.54.

*N-o-Nitrophenylsulfenyl-S-benzoyl-*L-*cysteine p-Nitrophenyl Ester.* N-*o*-Nitrophenylsulfenyl-S-benzoyl-L-cysteine DCHA salt (9.1 g., 0.016 mole) was transformed to the free acid by the procedure described above for the preparation of γ-*t*-butyl N-*o*-nitrophenylsulfenyl-L-glutamate. The sirupy acid thus obtained was dissolved in 60 ml. of ethyl acetate together with 2.65 g. of *p*-nitrophenol and 3.7 g. of N,N′-dicyclohexylcarbodiimide. After standing at room temperature for 2 hr., the dicyclohexylurea formed was removed by filtration and the filtrate was evaporated to dryness. Recrystallization of the residue from ethanol afforded 5.4 g. (67%) of the product, m.p. 101–102°, $[\alpha]^{25}D$ −51.4° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{22}H_{17}O_7N_3S_2$: C, 52.86; H, 3.43; N, 8.41; S, 12.83. Found: C, 52.80; H, 3.63; N, 8.80; S, 12.72.

*N,N′-Di-o-nitrophenylsulfenyl-*L-*cystinyl-diglycine ethyl ester* was prepared from N,N′-di-*o*-nitrophenylsulfenyl-L-cystine DCHA salt and glycine ethyl ester hydrochloride by the carbodiimide method in the same manner as described earlier for the preparation of N-*o*-nitrophenylsulfenyl-L-valyl-L-phenylalanine methyl ester (procedure b).[3] The crude crystalline product was recrystallized first from ethanol and then from ethyl acetate. The yield was 46%, m.p. 170–172°, $[\alpha]^{20}D$ −118.6° (*c* 3, dimethylformamide).

Anal. Calcd. for $C_{26}H_{32}O_{10}N_6S_4$: C, 43.56; H, 4.50; N, 11.72; S, 17.89. Found: C, 43.77; H, 4.78; N, 11.95; S, 18.06.

L-*Cystinyldiglycine Ethyl Ester Dihydrochloride.* A suspension of 3 g. (0.004 mole) of the corresponding N,N′-di-NPS derivative in 16 ml. of 2 *N* HCl in ethanol was shaken until a clear solution resulted. Upon the addition of dry ether, the above dihydrochloride precipitated as an amorphous powder. The yield was 85%, R_f 0.81 (thin layer chromatography[19] in 1-butol–acetic acid–water–pyridine[20]).

(19) M. Brenner and A. Niederwieser, *Experientia*, **16**, 378 (1960); A. R. Fahmy, A. Niederwieser, G. Pataki, and M. Brenner, *Helv. Chim. Acta*, **44**, 2022 (1961).

(20) S. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

Anal. Calcd. for $C_{14}H_{28}O_6N_4S_2Cl_2$: C, 34.79; H, 5.83; S, 13.25; Cl, 14.66. Found: C, 34.71; H, 5.63; S, 12.93; Cl, 14.35.

*S-Carbobenzoxy-N-o-nitrophenylsulfenyl-*L*-cysteinylglycine p-Nitrobenzyl Ester.* The free acid S-carbobenzoxy-N-NPS-L-cysteine, prepared from its DCHA salt as described above for the case of *t*-butyl N-NPS-L-glutamate, was coupled with glycine *p*-nitrobenzyl ester by the carboxylic–carbonic mixed anhydride method. The manner of coupling was described earlier (procedure C) for the preparation of N-NPS-L-valyl-L-phenylalanine methyl ester.[3] After recrystallization from ethyl acetate, the yield was 50%, m.p. 126–128°, $[\alpha]^{24}$D −46.0° (*c* 3, chloroform).

Anal. Calcd. for $C_{26}H_{24}O_9N_4S_2$: C, 51.99; H, 4.02; N, 9.32; S, 10.67. Found: C, 51.88; H, 4.31; N, 9.22; S, 10.54.

*S-Carbobenzoxy-*L*-cysteinylglycine p-Nitrobenzyl Ester Hydrobromide.* A suspension of 0.8 g. (0.0013 mole) of the corresponding N-NPS derivative in 10 ml. of 0.4 *N* HBr in ethanol was shaken until a clear solution resulted. Upon the addition of ether, 0.5 g. (73%) of the above hydrobromide was obtained, m.p. 141° after recrystallization from ethyl acetate–ethanol, $[\alpha]^{24}$D +18.4° (*c* 3, dimethylformamide); lit.[6] m.p. 140–141°; however the reported[6] $[\alpha]$D +13.3° was in error. It should had been written $[\alpha]$D +18.3°.

*S-Benzoyl-N-o-nitrophenylsulfenyl-*L*-cysteinylglycine methyl ester* was prepared in the same manner as the corresponding S-carbobenzoxy *p*-nitrobenzyl ester derivative. The yield was 46%, m.p. 139–140° after recrystallization from methanol, $[\alpha]^{22}$D +18.3° (*c* 3, chloroform).

Anal. Calcd. for $C_{19}H_{19}O_6N_3S_2$: C, 50.76; H, 4.26; N, 9.35; S, 14.27. Found: C, 50,82; H, 4.38; N, 9.25; S, 13.92.

*S-Benzoyl-*L*-cysteinylglycine methyl ester hydrochloride* was prepared by shaking a suspension of 0.45 g. (0.001 mole) of the corresponding NPS derivative in 4 ml. of 1 *N* HCl in methanol until a clear solution resulted. Upon the addition of ether, 0.25 g. (76%) was obtained, m.p. 163° after recrystallization from methanol–ethyl acetate, $[\alpha]^{23}$D +29.1° (*c* 5, methanol); lit.[6] m.p. 163–167°, $[\alpha]$D +29.0° (methanol).

*Benzyl N-o-Nitrophenylsulfenyl-γ-benzyl-*L*-glutamyl-*L*-glutaminate (I).* N-*o*-Nitrophenylsulfenyl-L-glutamic acid γ-benzyl ester DCHA salt[3] was transformed to the free acid, which, in turn was coupled with L-glutamine benzyl ester in the same manner as described earlier for the preparation of NPS-L-valyl-L-phenylalanine methyl ester (procedure A).[3] After recrystallization of the crude product from ethyl acetate, the yield was 75%, m.p. 139–140°, $[\alpha]^{21}$D −20.0° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{30}H_{32}O_8N_4S$: C, 59.20; H, 5.30; N, 9.20; S, 5.26. Found: C, 59.31; H, 5.59; N, 9.32; S, 5.02.

By coupling γ-benzyl α-nitrophenyl N-*o*-nitrophenylsulfenyl-L-glutamate[3] with L-glutamine benzyl ester the yield of recrystallized product was 35%, m.p. 139–140°.

*Benzyl N-o-Nitrophenylsulfenyl-*L*-glutaminyl-γ-benzyl-*L*-glutamyl-*L*-glutaminate (II).* Compound I (2.4 g., 0.004 mole) was dissolved in absolute acetone (20 ml.) by gentle heating. After the solution was cooled to room temperature, 3.6 ml. of 2 *N* HCl in ethanol was added and the solution was shaken for 3 min. Upon the addition of ether, 1.84 g. (93%) of benzyl γ-benzyl-L-glutamyl-L-glutaminate hydrochloride separated as a sirup (R_f 0.80, thin layer chromatography[19] in 1-butanol–acetic acid–water–pyridine[20]). In the meantime, 0.56 ml. of triethylamine and 1.04 g. of diphenylphosphoryl chloride[21] were added to a solution of 1.2 g. (0.004 mole) of NPS-L-glutamine[3] in 10 ml. of dimethylformamide, precooled at −5°. The mixture was kept at −5° for 15 min. and the mixed carboxylic–phosphoric anhydride which formed was added to a solution of 1.9 g. of the above sirupy dipeptide ester hydrochloride in 10 ml. of tetrahydrofuran and 1.12 ml. of triethylamine. After standing at room temperature for 3 hr., the solvent was removed *in vacuo*, the residue was taken up in ethyl acetate, and the resulting solution was washed with water, dilute potassium hydrogen carbonate, and again with water, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in warm ethyl acetate and the protected tripeptide ester was obtained, upon cooling, as an amorphous powder; the yield was 2 g. (70%), m.p. 174–177°, $[\alpha]^{25}$D −20.7° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{35}H_{40}O_{10}N_6S$: C, 57.05; H, 5.47; N, 11.40; S, 4.35. Found: C, 57.52; H, 5.55; N, 11.24; S, 4.26.

*Benzyl N-carbobenzoxy-*L*-phenylalanyl-*L*-glutaminyl-γ-benzyl-*L*-glutamyl-*L*-glutaminate (III).* The suspension of 3.7 g. (0.005 mole) of compound II in 30 ml. of 0.25 *N* HCl in ethanol was vigorously shaken until an almost clear solution resulted. After the addition of 30 ml. of ethyl acetate, traces of undissolved material were removed by filtration and ether was added to the filtrate. Thus, benzyl L-glutaminyl-γ-benzyl-L-glutamyl-L-glutaminate hydrochloride was precipitated as a sirup which solidified upon trituration with ether. This hydrochloride (R_f 0.78, thin layer chromatography[19] in 1-butanol–acetic acid–water–pyridine[20]) was coupled with carbobenzoxy-L-phenylalanine in chloroform solution in the same manner as described above for the preparation of II. The yield of III was 46% after recrystallization of the crude product from acetic acid–methanol; m.p. 246°, $[\alpha]^{20}$D −18.2° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{46}H_{52}O_{11}N_6$: C, 63.87; H, 6.06; N, 9.71. Found: C, 63.93; H, 6.26; N, 9.69.

*L-Phenylalanyl-*L*-glutaminyl-*L*-glutamyl-*L*-glutamine (IV).* Compound III (0.865 g., 0.001 mole) was suspended in 30 ml. of dimethylformamide and 0.3 ml. of acetic acid and catalytically (Pd) hydrogenated in the usual way. After the addition of 1 ml. of 1 *N* HCl, the catalyst was filtered and washed with water. The filtrate was adjusted to pH 5 by the addition of approximately 0.5 ml. of triethylamine. Upon adding acetone, peptide IV precipitated in the form of prisms. After recrystallization from water–acetone, the yield was 0.35 g. (77%), m.p. 233–235°, $[\alpha]^{20}$D −21.8° (*c* 1, 1 *N* HCl), R_f 0.16 (thin layer chromatography[19] in 1-butanol–acetic acid–water[12]), R_f 0.31 (descending

(21) A. Cosmatos, I. Photaki, and L. Zervas, *Chem. Ber.*, **94**, 2644 (1961).

chromatography on Whatman No. 1 paper, in 1-butanol–acetic acid–water–pyridine[20]).

Anal. Calcd. for $C_{24}H_{34}O_9N_6 \cdot H_2O$: C, 50.69; H, 6.38; N, 14.78. Found: C, 51.09; H, 6.29; N, 14.76.

The above tetrapeptide was completely hydrolyzed to phenylalanine, glutamine, and glutamic acid on incubation with leucine aminopeptidase, as was demonstrated by paper chromatography of the digest. Therefore, no racemization had taken place during the synthesis of IV.

Hydrogen Exchange in Chlorophyll and Related Compounds, and Correlation with Molecular Orbital Calculations[1]

Ralph C. Dougherty,[2] Harold H. Strain, and Joseph J. Katz

Contribution from the Chemistry Division, Argonne National Laboratory, Argonne, Illinois. *Received July 27, 1964*

Exchangeability of hydrogen at the C-10 and δ-methine positions in chlorophyll, bacteriochlorophyll, and some of their derivatives has been compared in several solvent systems. With methanol in tetrahydrofuran and in acetone, the hydrogen situated at both positions is labile, but exchange at C-10 is at least two orders of magnitude faster than at the δ-position. With methanol in pyridine, hydrogen exchange at C-10 is very rapid, whereas exchange at all methine carbon atoms is very slow. Exchange at the δ-position is influenced by the presence of magnesium; removal of the magnesium reduces the exchange rate at the bridge positions to a very low value. The experimental observations are discussed in the light of semiempirical molecular orbital calculations based on porphin and chlorin compounds.

Introduction

The photosynthetic role of chlorophyll as a hydrogen donor in a reversible cycle has long been a subject for speculation.[3] Such a hypothesis implies exchangeable hydrogen either in the ground state or excited states of chlorophyll. Consequently, a number of investigations have been carried out over the past 30 years in a search for labile hydrogen in chlorophyll. The first positive indication of "active" hydrogen in chlorophyll was provided by Fischer and Goebel[4] who used the Zerewitinoff reaction with methylmagnesium iodide in pyridine solution. They found one carbon-bound active hydrogen atom per mole in a large series of chlorophyll derivatives. Reaction conditions involving this use of the Grignard reagent are necessarily severe, and subsequent efforts to detect labile hydrogen in chlorophylls *a* and *b* and pheophytin *a* by exchange with tritium oxide[5] or deuterium oxide[6] were unsuccessful The more recent tritium studies of Vishniac and co-workers,[7] however, suggested that hydrogen exchange between water and chlorophyll occurs during photosynthesis, but no direct information about the site of exchange could be obtained. Russian investigators have been particularly concerned with hydrogen exchange in chlorophyll and have carried out extensive research in this area.[8–11] The reactivity of chlorophyll in hydrogen exchange has been clarified by the application of infrared and proton magnetic resonance spectroscopy, and, although the results by themselves neither support nor deny the hypothesis of a chemical role for chlorophyll in photosynthesis, the presence of labile hydrogen in chlorophyll has now been firmly established.

Chlorophylls *a* and *b* have previously been shown by an infrared procedure to exchange one proton with a stoichiometric equivalent of CH_3OD in carbon tetrachloride solution.[12] Preliminary nuclear magnetic resonance measurements suggested that this proton was located at the δ-methine position.[13] A detailed examination of the n.m.r. spectra of chlorophyll and its derivatives, however, indicated that the resonances from these compounds are subject to remarkable solvent and concentration effects.[14] The early n.m.r. observations, indicating that only the δ-hydrogen undergoes exchange, were made under conditions that virtually precluded observation of the C-10 proton resonance because of the "aggregation broadening" in pure $CDCl_3$ solutions. When exchange of hydrogen with chlorophyll was investigated under conditions that allowed direct and unambiguous observation of both the δ and C-10 resonances, it was found that both protons do in fact undergo exchange in neutral solutions, with the C-10 exchange about two orders of

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.

(2) Resident Research Associate.

(3) A. A. Krasnovsky, *Ann. Rev. Plant Physiol.*, **11**, 363 (1960).

(4) H. Fischer and S. Goebel, *Ann.*, **522**, 168 (1936).

(5) T. H. Norris, S. Ruben, and M. B. Allen, *J. Am. Chem. Soc.*, **64**, 3037 (1942).

(6) J. Weigl and R. Livingston, *ibid.*, **74**, 4160 (1952).

(7) B. Coleman and W. Vishniac, *Natl. Acad. Sci.–Natl. Res. Council Publ.*, **No. 1145**, 213 (1963); W. Vishniac and I. Rose, *Nature*, **182**, 1089 (1958).

(8) A. N. Sidorov, *Opt. i Spektroskopiya*, **13**, 374 (1962); **13**, 206 (1962); **14**, 834 (1963).

(9) V. M. Kutyurin, A. V. Karyakin, A. K. Chibisov, and I. Yu. Artamkina, *Dokl. Akad. Nauk. SSSR*, **141**, 744 (1961).

(10) A. J. Karyakin and A. K. Chibisov, *Opt. i Spektroskopiya*, **13**, 379 (1962).

(11) A. N. Sidorov and A. M. Terenin, *ibid.*, **8**, 259 (1960).

(12) J. J. Katz, M. R. Thomas, H. L. Crespi, and H. H. Strain, *J. Am. Chem. Soc.*, **83**, 4180 (1961).

(13) J. J. Katz, M. R. Thomas, and H. H. Strain, *ibid.*, **84**, 3587 (1962).

(14) G. L. Closs, J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, *ibid.*, **85**, 3809 (1963).